Hepatic Estrogen Binding During Vitellogenesis in Pacific Hagfish *Eptatretus stouti*

Walton W. Dickhoff, Stacia A. Sower and Aubrey Gorbman
Department of Zoology, University of Washington, Seattle, USA

During the reproductive phase of oviparous vertebrates, the liver synthesizes egg yolk precursors which are released into the circulation to be taken up by the growing egg and deposited as yolk. In gnathostomes, the hepatic production of yolk precursors (vitellogenin) is under the influence of estrogens. In the river lamprey, there is also evidence that estrogen stimulates vitellogenesis.[1] Recently, we extended the list of vertebrates in which estrogen stimulates hepatic synthesis of vitellogenin to include another agnathan species, the Pacific hagfish.[2,3]

A model of estrogen action on sensitive tissues has been derived from studies of the chicken oviduct and rat uterus. High affinity, low capacity estrogen binding proteins present in the cell cytoplasm are translocated to the nucleus in the presence of estrogen. We have demonstrated the existence of a single class of high affinity, low capacity estrogen binding sites in hepatic nuclei of hagfish.[4] The concentration of these nuclear binding sites is highest in vitellogenic hagfish. The purpose of the present study is the characterization of possible cytoplasmic binding of estrogen, which may be the progenitor of the nuclear estrogen binding component found in vitellogenic hagfish.

Pacific hagfish (*E. stouti*) were housed in covered aquaria and maintained unfed at 11°C in aerated seawater for 1 to 4 months until utilized. Adult females, with body lengths from 25 to 60 cm, were used. Females with eggs measuring greater than 5 mm (longest diameter) were considered vitellogenic.[5] For most assays livers from 2 vitellogenic and 2 previtellogenic females were pooled.

Hagfish were anesthetized by immersion in 0.6% MS 222, and blood was collected from the caudal sinus. After laparotomy, the liver was dissected from connective tissue. Each of the two hepatic lobes were perfused with 30 ml of TED buffer (10 mM Tris-HCl, 1.5 mM EDTA, 2 mM dithiothreitol at pH 7.4). Excess connective tissue was trimmed and the liver was placed in TED buffer on ice. All dissections were done at 10°C.

Tissues (500 mg/ml) were homogenized with 15 strokes in ice-cold TED using a motor driven glass-Teflon homogenizer. The homogenate was centrifuged at 2000 *g* for 15 min at 4°C. The supernatant was centrifuged at 100,000 *g* for 60 min at 4°C to obtain the cytosol preparation. To remove endogenous steroid, cytosol was treated with 1/10 vol of dextran-coated charcoal (0.5% dextran, 5% charcoal) for 7 min at 0°C before centrifuging twice at 2000 *g* for 10 min each time. Cytosol was diluted with TED to 150 to 200 mg equivalent wet weight of tissue per ml (1 mg protein/ml). For incubation, 1 ml of cytosol or plasma was dispensed into 10 × 75 mm glass tubes. H^3-estradiol (E_2; sp act 102 Ci/mM) in ethanol was added in a volume of 10 μl. Radioinert steroid in ethanol or ethanol alone was added in 10 μl volume. The tubes were mixed by vortexing and then incubated at 10°C for 1h.

After incubation the tubes were placed in an ice bath for 10 min before 0.5 ml of hydroxylapatite (HAP) was added. HAP (Bio-Rad Biol-Gel HT hydroxylapatite) was prepared by several washes with 0.5 M Tris-HCL, 0.001 M KH_2PO_4 buffer until the wash was pH 7.2 at 0°C. Incubation tubes containing HAP were mixed every 5 min for 30 min at 0°C. The tubes were centrifuged at 800 *g* for 10 min at 4°C. The pellet was washed 3 times with 2 ml TK (0.5 M Tris-HCL, 0.001 M KH_2PO_4) buffer. After the final wash 2 ml of ethanol was added to the pellet and the tubes were allowed to stand overnight at room temperatures (21°C). The tubes were centrifuged and the supernatant was poured into 10 ml scintillation fluid and counted on a Beckman liquid scintillation counter at 23% efficiency.

Total binding of $(H^3)E_2$ to cytosol was

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measured at concentrations of $(H^3)E_2$ ranging from 0.3 to 25 nM (1 ml volume). The nonspecific binding (NSB) component was determined for each concentration of $(H^3)E_2$ by prior addition of a 100-fold molar excess of radioinert E_2. Specific binding was determined by substracting NSB from the total binding. The apparent dissociation constant (K_d) was determined by Scatchard analysis.

A representative saturation curve for liver cytosol is shown in Fig. 1. NSB was linear throughout the concentration range of $(H^3)E_2$. Specific binding shows saturation between 12 and 25 nM $(H^3)E_2$. In contrast, our previous study of E_2 binding to liver nuclei showed saturation between 1.5 and 2.5 nM E_2. Scatchard analysis of these data to determine the apparent affinity of H^3E_2 for the cytosolic binding sites is shown in Fig. 1. It is not clear from these data whether there is one or two classes of binding sites. Dissociation constants of 5.9×10^{-9} M and 1.8×10^{-8} M could be calculated. Since it is possible that binding sites could have been destroyed in the presence of low concentrations of ligand, additional studies using near-saturation concentrations of $(H^3)E_2$ should be performed. Neither of the apparent K_ds of the cytosolic binding component are as high as that of the nuclear component (3.8×10^{-10}). These results suggest that the cytosolic binding component may have a lower affinity for E_2 than does the nuclear component.

Further characterization of the cytosolic binding component involved studies of binding specificity. Specificity of binding was carried out using 10 nM $(H^3)E_2$ and 10- to 20,000-fold molar excess of radioinert competitor. Estrone (E_1) was equally active as E_2 in displacing $(H^3)E_2$ from the cytosolic component. Diethylstilbesterol (DES) was one-tenth as active as E_2 while estriol (E_3) was one-hundredth as active. Progesterone and testosterone were poor competitors for cytosolic E_2 binding. Previous studies of the specificity of nuclear binding showed that E_1, E_2, E_3, and DES had approximately equal binding activities. These results indicate that the specificity of estrogen binding to the cytosolic component differs from that of the nuclear component.

It is unlikely that cytosolic binding of steroids was due to contamination of the cytosol preparation with plasma, since the livers were perfused and rinsed with buffer. Further verification of the lack of such plasma contamination was provided by specificity studies of plasma steroid binding. Incubation of 0.5% plasma with 10 nM $(H^3)E_2$ was carried out in a manner similar to that of cytosol. Radioinert steroids were added in concentrations of 50- to 200-fold molar excess. The results indicated that DES and E_1 were the most potent competitors for $(H^3)E_2$ binding to the plasma component. E_2 and E_3 were one-sixth as effective as E_1. Progesterone and testosterone were poor

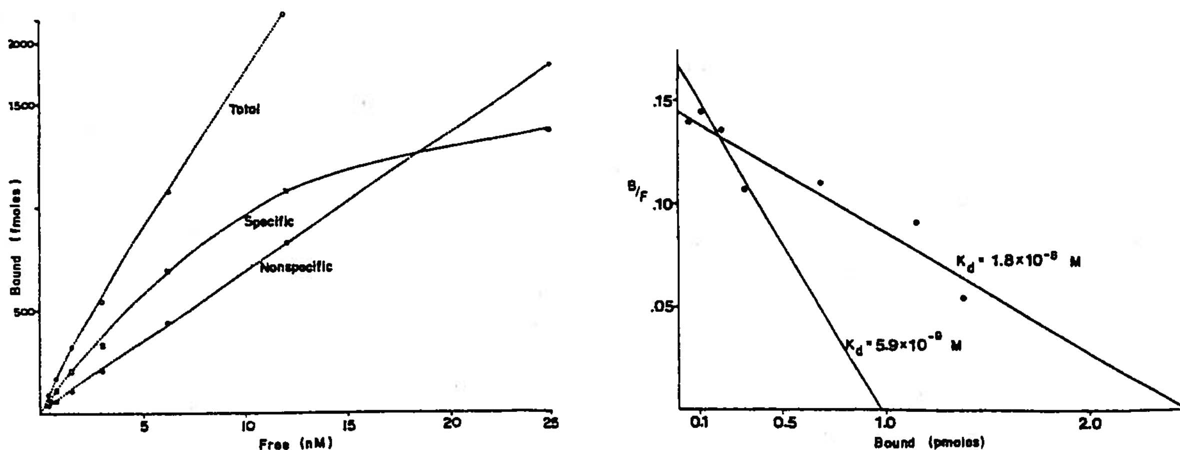

Fig. 1. A representative saturation analysis *(left)* and Scatchard plot *(right)* of estradiol binding to liver cytosol (1 mg protein/ml). Each point represents the average of three replicate samples. The specific binding curve was determined by subtracting the nonspecific binding from the total binding. In the Scatchard plot the apparent K_d were calculated as -1/slope. There may be one or two binding components in the cytosolic preparation.

competitors. These results suggest that the specificity of steroid binding of the plasma component differs from both that in the cytosol and that in the nucleus.

The cytosolic binding component of hagfish liver differs from that of the nucleus on the basis of both apparent affinity and steroid binding specificity. Furthermore, the cytosolic component differs from steroid binding activity in hagfish plasma. It is unlikely, therefore, that the cytosolic component is the nuclear hormone receptor waiting to be translocated into the nucleus according to the model of steroid hormone action derived from studies of higher vertebrates. A cytosolic steroid binding component in liver cytosol of the chicken has been shown to differ in its affinity for steroid hormones.[6] The binding protein in chicken liver cytosol has been called 'hepatic steroid-binding protein' or HSBP. One of the functions ascribed to HSBP is the maintenance of a relatively constant intracellular level of estrogen during fluctuations of plasma estrogen levels. It would be significant if the hagfish had a similar mechanism since plasma levels of E_2 in the hagfish are low and highly variable. On the other hand, the cyctosolic binding of hagfish liver has a capacity of 1 to 2 pmoles per mg protein which is one hundred times less than that of HSBP in chicken liver.

The possibility of a fish HSBP has been suggested recently by van Bohemen *et al.*[7] These authors found that injections of E_1 into trout was much more effective in inducing vitellogenesis than could be accounted for by the intrinsic activity of E_1. Displacement of E_2 by E_1 from a plasma or cytoplasmic estrogen binding component could explain these observations.

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